We sincerely thank the reviewers for their constructive comments on our manuscript. We have incorporated their suggestions into a revised version of the manuscript that we believe is much improved.

Comments from Reviewer #1

Major Concerns

1) **The last step of the new methodology is missing. Identification by LC-MS/MS of putative CK2 substrates in at least one unique band should be illustrated.**
   This is a critical point (also raised by Reviewer #3) and we have now included data in a new figure (Figure 4) demonstrating that several previously identified CK2 substrates as well as some potential novel substrates were indeed identified using the described method. In addition, we have included confirmation by Western blot analysis for one known CK2 substrate protein (nucleolin), demonstrating that it was specifically thiophosphorylated and subsequently immunoprecipitated.

2) **The reviewer questions to what extent endogenous CK2 contributes to the overall phosphorylation of endogenous substrates and suggests that this contribution may vary depending on the tissue/cell type used. Likewise, it is expected that free -SH groups will be alkylated and contribute to the cross-reactivity disclosed in Figure 2.**
   The reviewer is correct on both accounts. It is almost certain that endogenous CK2 contributes to the phosphorylation of endogenous substrates and that this likely varies between cell and tissue types. Furthermore, as the reviewer surmises, the background signal observed in Figure 2 is likely due to alkylation of free thiol groups on various proteins.

3) **The reviewer is concerned whether previous/variable occupancy of CK2 phospho-sites interferes with the methodology and asks whether dephosphorylation of proteins prior to the addition of exogenous CK2 was attempted in order to determine if the results of the experiment would be altered.**
   While we have not directly tested if dephosphorylation of proteins prior to the addition of CK2 would change the outcome of the experiment, we predict (as probably the reviewer does as well) that it would. This is therefore a drawback to the methodology and may result in some CK2 substrates not being identified via this strategy. We have added a sentence acknowledging this limitation in the revised Discussion section. This same comment was also made by Reviewer #3.

4) **In the Discussion section, an important limitation of the new methodology should be highlighted: the identified thiophosphorylated proteins are thus far only in vitro substrates of CK2. The actual occurrence of their phosphorylation in vivo remains to be demonstrated.**
   We agree with the reviewer, and we suggest that this approach simply provides an initial platform for identifying potential novel CK2 substrates. Subsequent validation of these proteins as *bona fide* CK2 substrates in cell-based or in-vivo assays is absolutely essential and we have highlighted this point in the Discussion section.

Minor Concerns

It would be advisable to include a list of abbreviations used.

We have made sure to fully define all abbreviations the first time they are used in the manuscript.

While mentioning previous studies using quantitative phosphoproteomics approaches, a reference describing the strategy of knocking out both CK2 catalytic subunits (Franchin *et al*., 2018, *Cell. Mol. Life Sci.*) should also be cited.
We thank the reviewer for catching this omission. The suggested additional reference is now cited appropriately within the manuscript and in the References section.

Comments from Reviewer #3

Major Concerns

1) **The reviewer states that the results, as shown, are promising but preliminary. The overall impact of this manuscript is diminished because it is unclear whether the method, as presented, has been used successfully to identify CK2 substrates (either known or novel) and whether sufficient protein has been thiophosphorylated and isolated to enable identification by mass spectrometry.**
   This is definitely an important point, shared by Reviewer #1 (see comment 1 above), and we believe this concern is now adequately addressed with the inclusion of the data in Figure 4.

2) **The reviewer comments that certain aspects of the experimental procedure are described qualitatively instead of quantitatively. For instance, the amount of CK2 enzymatic activity should be indicated rather than listing the volume of CK2 enzyme added to the reaction.**
   We have now listed the activity of the enzyme (in U) that corresponds to the volume of CK2 used in the kinase reactions.

3) **To ensure that the substrates identified are physiologically relevant substrates of CK2, the manuscript should consider validation strategies that include the demonstration that sites of thiophosphorylation are actual phosphorylation sites in living cells (and are not simply sites that are phosphorylated when there is an excess of CK2 in a cell lysate).**
   This is also a concern expressed by Reviewer #1 (see comment 4 above). These types of validation studies are now highlighted in the Discussion section as being essential follow-up experiments to demonstrate that an identified substrate of CK2 is physiologically relevant.

4) **Similar to point #3, the utility of this method would be increased if the authors could demonstrate that specific sites of thiophosphorylation/phosphorylation could be identified.**
   We likewise agree and in the Discussion section propose identification of specific CK2 phosphorylation sites as logical next steps in the workflow.

5) **The reviewer comments that, given the constitutive activity of CK2, it is possible that a number of CK2 substrates will not be identified using this strategy since the physiologically relevant sites will already be fully occupied in cell lysates.**
   The reviewer is absolutely correct, and this issue was also raised by Reviewer #1 as a potential drawback of the approach (see comment 3 above). We have therefore added a sentence to the Discussion section that describes this particular scenario as a limitation of the methodology.

Minor Concerns

Column Preparation: It would be helpful to the reader to identify the specific resin that is used here.

The type of resin (Sephadex G-25 desalting resin) is now indicated.

Figure 2: It is unclear why all lanes show relatively similar levels of CK2 by immunoblotting since excess CK2 was not added to all lanes.

This discrepancy is most likely due to a dilutional effect. The kinase was diluted 100-fold in the reaction volume, and then only a small fraction of this was loaded onto the gel. The anti-CK2α antibody may not be sensitive enough to detect this minor difference in protein levels.

Table of Materials: What is the concentration of the stock lysis buffer?

The recipe listed in the table is to generate 10 ml of 1X lysis buffer.